AMNIOTIC FLUID BILIRUBIN MEASUREMENT: A COMPARISON OF FIVE METHODS

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Introduction

Since Bevis (1) first showed a correlation between the severity of hemolytic disease of the newborn and the amounts of several substances present in the amniotic fluid, amniocentesis has been widely used as a diagnostic aid in this disease. Liley (13, 14) showed that the most important of these substances was bilirubin, which he measured using a direct spectrophotometric method. Results were expressed in optical density units.

Although most workers since then have been satisfied with Liley's method, others have attempted to quantitate bilirubin levels in amniotic fluid. Robertson (19), Bower and Swale (2), Watson and Mackey (22), and Freda (8) used various diazotization techniques for this purpose. Fleming and Woolf devised another direct spectrophotometric method, which unlike Liley's was quantitated to give results in mg. per cent of bilirubin (5).

This paper will compare several of these methods for measuring bilirubin, in terms of accuracy, reproducibility and convenience.

Method

After localization of the placenta with radioactive ^{131}I labeled albumin, amniotic fluid was obtained by transabdominal percutaneous puncture with a 20 gauge spinal needle. Ten to fifteen cc. of amniotic fluid was withdrawn and centrifuged for 30 minutes at 3000 rpm. If still cloudy, it was then filtered through Whatman No. 1 paper with suction.

Method I:

The ΔO.D. (Liley) was determined by measuring the optical density of the amniotic fluid against distilled water from 365 mμ to 700 mμ in a continuous recording Perkin-Elmer 202 Spectrophotometer using 1 cm. path length cells (13). The tangent to the curve at approximately 365 mμ and 550 mμ was drawn and height of the curve from the tangent at 450 mμ was expressed in optical density units.

Method II:

Bilirubin was measured by the method of Fleming and Woolf (5) in the following manner. Using distilled water as a blank, the absorbance of the amniotic fluid was measured in 1 cm. path length cells at 700 mμ, 623 mμ , 576 mμ, and 462 mμin the Zeiss PM QII/M4 QII Spectrophotometer. Fleming and Woolf described the addition of a few crystals of sodium dithionite to the amniotic fluid, repeating the reading at 700 mμ, and using the lower of the two readings at this wavelength. This author, however, has found that addition of sodium dithionite invariably increases the absorbance at 700 mμ, and therefore this chemical was not added. Bilirubin, in mg./100 ml., was calculated using the formula: Bilirubin = 1.25D - 0.91C - 1.15B + 0.11A, where A = O.D. at 700 mμ, B = O.D. at 623 mμ, C = O.D. at 576 mμ, and D = O.D. at 462 mμ.

Method III:

Bilirubin was then measured by the method of Robertson (19).

Reagents:

(1) Diazo A: Sulfanilic acid, 1 gm. in 1000 ml. of hydrochloric acid solution prepared by mixing 15 ml. of concentrated HCl with water to make 1000 ml.

Diazo B: Sodium nitrite 0.5%, made fresh daily by adding 0.1 ml. of 50% stock solution of sodium nitrite to 9.9 ml. of water. The sodium nitrite stock solution is kept refrigerated.

Diazo solution: Add 0.3 ml. of solution B to 10 ml. of solution A. Make fresh daily.

(2) Hydrochloric acid solution: Mix 15 ml. of concentrated HCl to water to make 1000 ml.

(3) Methanol

Method:

The following solutions were prepared:

(A) 2 ml. amniotic fluid, 1 ml. diazo solution, and 3 ml. HCl solution.

(B) 2 ml. amniotic fluid and 4 ml. HCl solution.

(C) 2 ml. amniotic fluid, 1 ml. diazo solution, and 3 ml. methanol.

(D) 2ml. amniotic fluid, 3 ml. methanol, and 1 ml. HCl solution.

All solutions were allowed to stand for 10 minutes. Solution B was read against A to determine direct bilirubin, and D against C to determine total bilirubin.

All optical density measurements were made in 1 cm. path length cuvettes at 530 mμ in the Zeiss PM QII/M4 Spectrophotometer.

Robertson did not describe preparation of standards, but they can be prepared by adding commercial bilirubin in chloroform solution to methanol and substituting for methanol in solutions C and D.

Method IV:

Bilirubin was then measured by the method of Bower and Swale (2).

Reagents:

(1) Diazo reagent: Solution A: 1 gm. sulfanilic acid in 1000 ml. of 0.175 N HCl. Solution B: 0.5% sodium nitrite, prepared as described above.

Ten ml. of A was mixed with 0.3 ml. of B. After 5 minutes 1 ml. of 1.5% ammonium sulfamate was added, and the reagent was used after standing for at least 3 minutes.

Blank reagent was made by substituting distilled water for solution B in the diazo reagent.

(2) Ethanol-urea solution: 25 gm. of urea was dissolved in 100 ml. of 85% (v/v) ethanol by heating. The solution was filtered through glass wool while still warm and kept in an incubator to prevent crystallization of urea.

(3) Sodium azide, powder.

(4) Phenol, liquid, 88% (Mallinkrodt).

(5) Neutral buffer: 5.0 gm. of sodium citrate was dissolved in distilled water and made up to 100 ml.

(6) Acid buffer: 11.8 gm. of sodium citrate was dissolved in 0.2 N HCl to a volume of 200 ml. and this was made up to 500 ml. with distilled water.

(7) Chloroform

Method:

Amniotic fluid, 0.5 ml., was put in each of two 10 ml. test tubes. To the first was added 0.5 ml. of diazo reagent, and to the second, 0.5 ml. of blank reagent. Both tubes were placed in a beaker containing ice-colled water. Ethanol-urea, 2.2 ml., and 0.2 ml. of chloroform was added, and the tubes were mixed and allowed to stand in the ice water for 10 minutes. After removal from the ice, a tiny fragment of sodium azide was added to each tube and dissolved by shaking. Neutral buffer, 0.1 ml., was added followed by mixing. Phenol, 2.5 ml., was added by burette into each tube and the tubes were vigorously mixed with a Vortex-Genie mixer. The diazo mixture was read against the blank at 530 mμ in 3 cm. path length cuvettes using the Zeiss PM Q11/M4 Q11 Spectrophotometer.

Standards were prepared from bilirubin 1 mg./ml. in chloroform (Fisher). This was used in the reaction mixture in place of chloroform, and acid buffer was used instead of amniotic fluid. Appropriate dilutions of the bilirubin standard were made in chloroform to give bilirubin concentrations equivalent to 2.0, 1.0, 0.5, 0.25, and 0.125 mg./100 ml. in amniotic fluid.

Method V:

Bilirubin was then measured by the Jendrassik-Grof method as described by Gambino (9). Proportions of the reagents have been changed to make the resulting color more intense.

Reagents:

(1) Caffeine mixture: 50 gm. of caffeine, 75 gm. of sodium benzoate, and 125 gm. of sodium acetate ($\cdot 3H_2O$) were added to distilled water at 50-60°C., and the volume was brought up to 1000 ml. when cool. It was then filtered through glass wool.

(2) Diazo A: 5.0 gm. sulfanilic acid and 15.0 ml. of concentrated hydrochloric acid were added together and the volume brought up to 1000 ml. It was then filtered through glass wool.

Diazo B: 0.5% sodium nitrite solution was prepared as described above.

Diazo reagent was prepared by mixing 10.0 ml. of Diazo A with 0.25 ml. of Diazo B. Diazo reagent was made fresh daily.

(3) Alkali mixture: 100 gm. of sodium hydroxide and 350 gm. of potassium sodium tartrate were dissolved in distilled water and the volume brought up to 1000 ml. It was filtered through glass wool.

Method:

Caffeine mixture, 1.0 ml., was added to each of 2 tubes, labeled unknown and blank. To each tube was added 0.5 ml. of amniotic fluid. After the addition of 0.17 ml. of Diazo reagent to the unknown tube and 0.17 ml. of Diazo A to the blank, both tubes were mixed and allowed to stand for 10 minutes, after which 0.5 ml. of alkali mixture was added and the tubes again shaken. Within 30 minutes, the optical

density of the unknown was measured against the blank, using 1 cm. light path cuvettes in the Zeiss PM QII/M4 QII spectrophotometer.

Standards were prepared as follows. Bilirubin powder, 20 mg., (Eastman) was weighed to within 0.1 mg. in a tiny aluminum foil "boat" which was then pushed to the bottom of a 100 ml. volumetric flask so that all the bilirubin was deposited at the bottom of the flask. In subdued light 4 ml. of 0.1 N NaOH was added and the bilirubin dissolved by swirling. Fifty ml. of 1.5 gm. per cent human serum albumin in normal saline, prepared from pooled concentrated human serum albumin (American National Red Cross), was then added and the contents of the flask again swirled. Four ml. of 0.1 N HCl was added to neutralize the alkali, and 1.5% albumin was added to bring the volume up to 1000ml. Using the albumin solution and saline, solutions of 2.0, 1.0, 0.5, 0.25, and 0.10 mg. per cent bilirubin in 0.75 gm. per cent albumin were prepared and subjected to diazotization in the same manner as amniotic fluid.

The following tests were performed on amniotic fluid samples:

ΔO.D. (Liley)	-	all samples
Bilirubin (Fleming - Woolf)	-	85 samples
Bilirubin (Bower - Swale)	-	58 samples
Bilirubin (Robertson)	-	20 samples
Bilirubin (Jendrassik - Grof)	-	51 samples

The values obtained by each method were plotted as the abcissa versus the corresponding ΔO.D. (Liley) values as ordinates. The best straight line was drawn through these points as calculated by the following formula:

Equation of line is $Y = \alpha + \beta X$ where

$$\alpha = \frac{\Sigma Y \Sigma X^2 - \Sigma X \Sigma XY}{N\Sigma X^2 - (\Sigma X)^2}$$

$$\beta = \frac{N\Sigma XY - \Sigma X \Sigma Y}{N\Sigma X^2 - (\Sigma X)^2}$$

The coefficient of correlation (r) was calculated as follows:

$$r = \frac{N\Sigma XY - \Sigma X \Sigma Y}{\sqrt{\{N\Sigma X^2 - (\Sigma X)^2\}\{N\Sigma Y^2 - (\Sigma Y)^2\}}}$$

The standard error of estimate is found by the following calculation:

$$s = \sqrt{\frac{\Sigma Y^2 - (\alpha \Sigma Y + \beta \Sigma XY)}{N}}$$

The standard deviation of repeated measurements made on the same sample is: $\sigma = \sqrt{\frac{\Sigma x^2}{N}}$, where x is the deviation of each measurement from the mean and N is the number of samples.

For the Jendrassik-Grof method the molar absorptivity of alkaline azobilirubin was calculated from this equation:

$$M.A.\,{}^{600m\mu}_{1cm.} = \frac{\text{M.W.} \cdot \text{A/cm.} \cdot \text{final volume in ml.}}{\text{Total amount of bilirubin in mg.}}$$

Where $M.A.\,{}^{600m\mu}_{1cm}$ = Molar absorptivity at 600 mμ for 1 cm. light path, M.W. = Molecular weight of bilirubin (584), A/cm. = Absorptivity per cm. of light path.

As suggested by Gambino (9), the molecular weight of bilirubin and not azobilirubin is used in the calculation.

Results

The results are summarized in Table 1

Table 1

Method: all versus ΔO.D. (Liley)	Equation of best straight line	Coefficient of correlation	Standard error of estimate (Optical density units)	Standard deviation of repeated measurements on a single sample (mg.% bilirubin)
Bower-Swale	ΔO.D.= 0.014+0.66Bili	0.94	0.046	0.043
Fleming-Woolf	ΔO.D.= -0.016+0.59Bili	0.96	0.034	0.01
Jendrassik-Grof	ΔO.D.= -0.025+0.74Bili	0.99	0.024	0.021
Robertson	Unusable (See text)	--	--	--

Bilirubin was measured by the Fleming-Woolf method in 85 samples and the comparison between these values and the ΔO.D. (Liley) is shown in Figure 1.

The Bower-Swale method was used to measure bilirubin in 58 samples, and the plot against ΔO.D. is shown in Figure 2. The standardization curve for the Bower-Swale method is shown in Figure 3.

Bilirubin in 51 samples was measured using the Jendrassik-Grof method and the results are plotted against ΔO.D. in Figure 4. The standardization curve is shown in Figure 5. The molar absorptivity of azobilirubin is calculated to be 72,500.

In all graphs the dashed lines represent ±1 standard error of

estimate.

Robertson's method for measuring total bilirubin gave solutions with such a great amount of turbidity that they could not be read in the spectrophotometer, and therefore it could not be used. The direct bilirubin was negligible in the 20 samples tested, and therefore the indirect and total bilirubin were nearly equal.

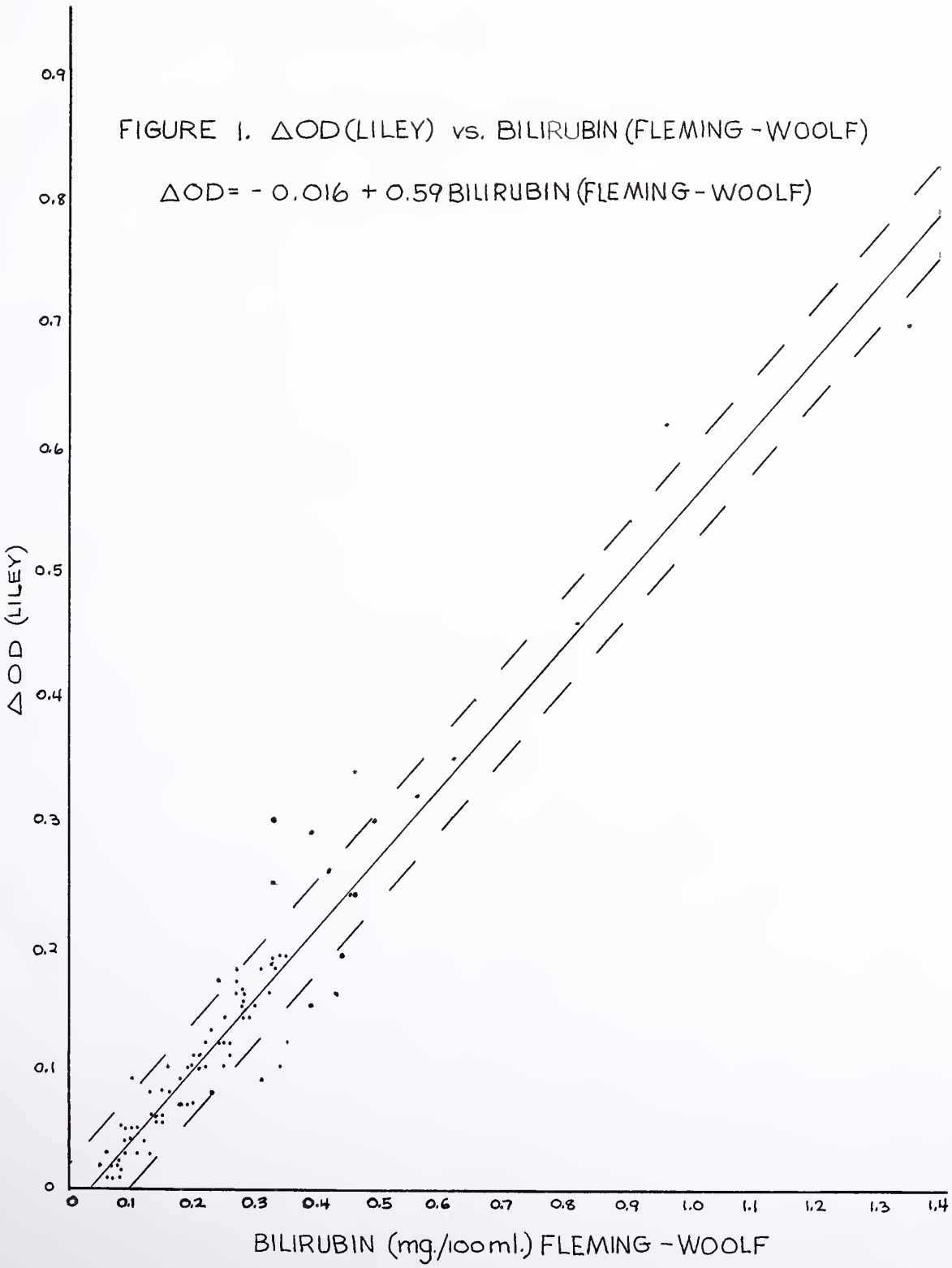
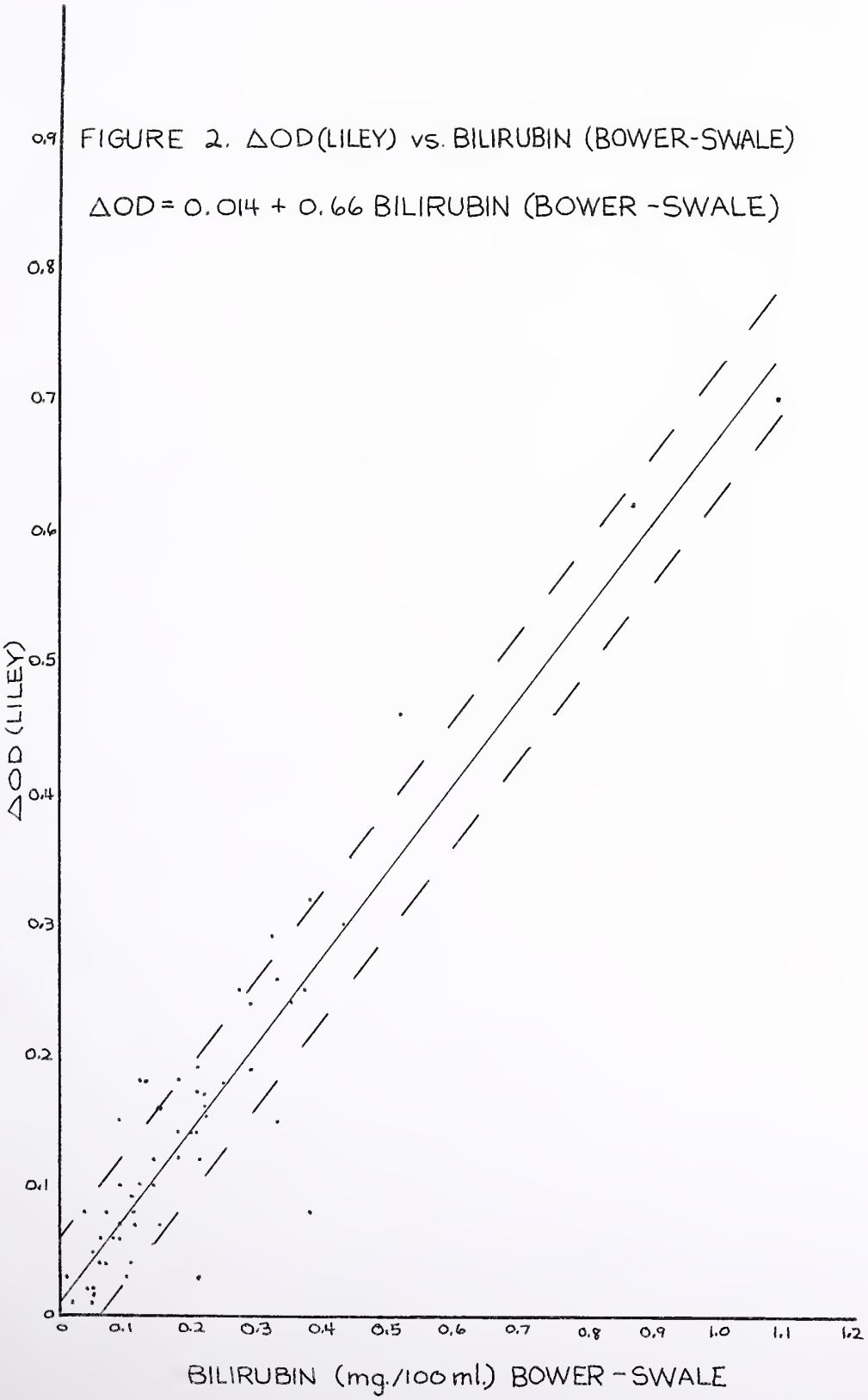


FIGURE 1. ΔOD (LILEY) vs. BILIRUBIN (FLEMING - WOOLF)
ΔOD = - 0.016 + 0.59 BILIRUBIN (FLEMING - WOOLF)
ΔOD (LILEY)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
BILIRUBIN (mg./100ml.) FLEMING - WOOLF

FIGURE 2. ΔOD (LILEY) vs. BILIRUBIN (BOWER-SWALE)

ΔOD = 0.014 + 0.66 BILIRUBIN (BOWER - SWALE)



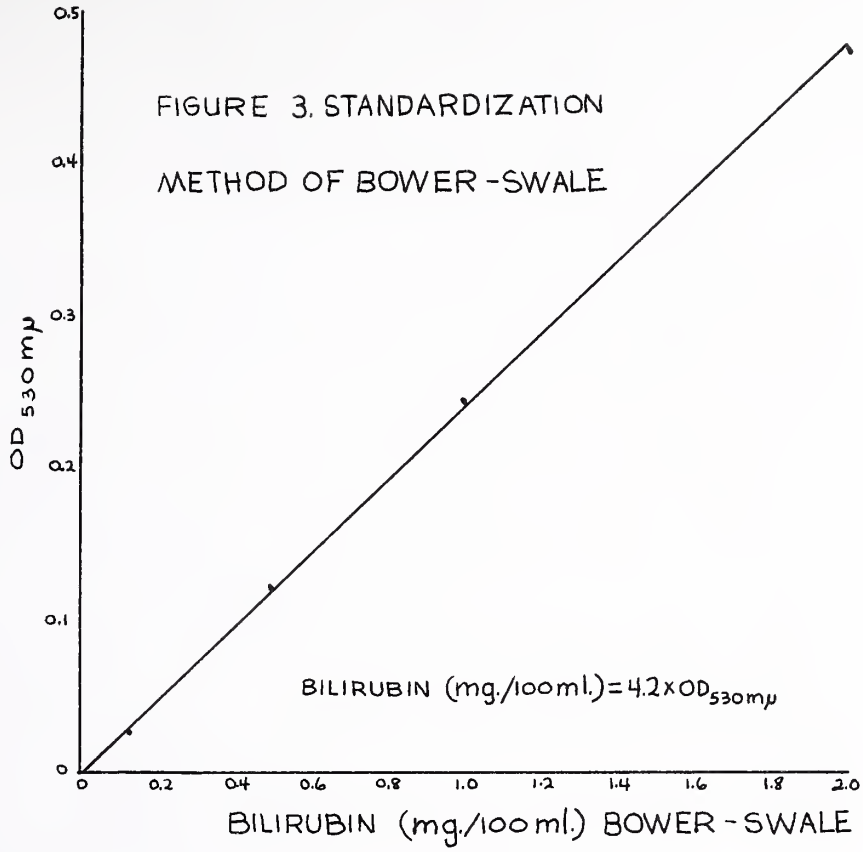


FIGURE 3. STANDARDIZATION
METHOD OF BOWER-SWALE
OD 530 mμ
0.5
0.4
0.3
0.2
0.1
0
BILIRUBIN (mg./100ml.)=4.2xOD530mμ
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
BILIRUBIN (mg./100 ml.) BOWER-SWALE

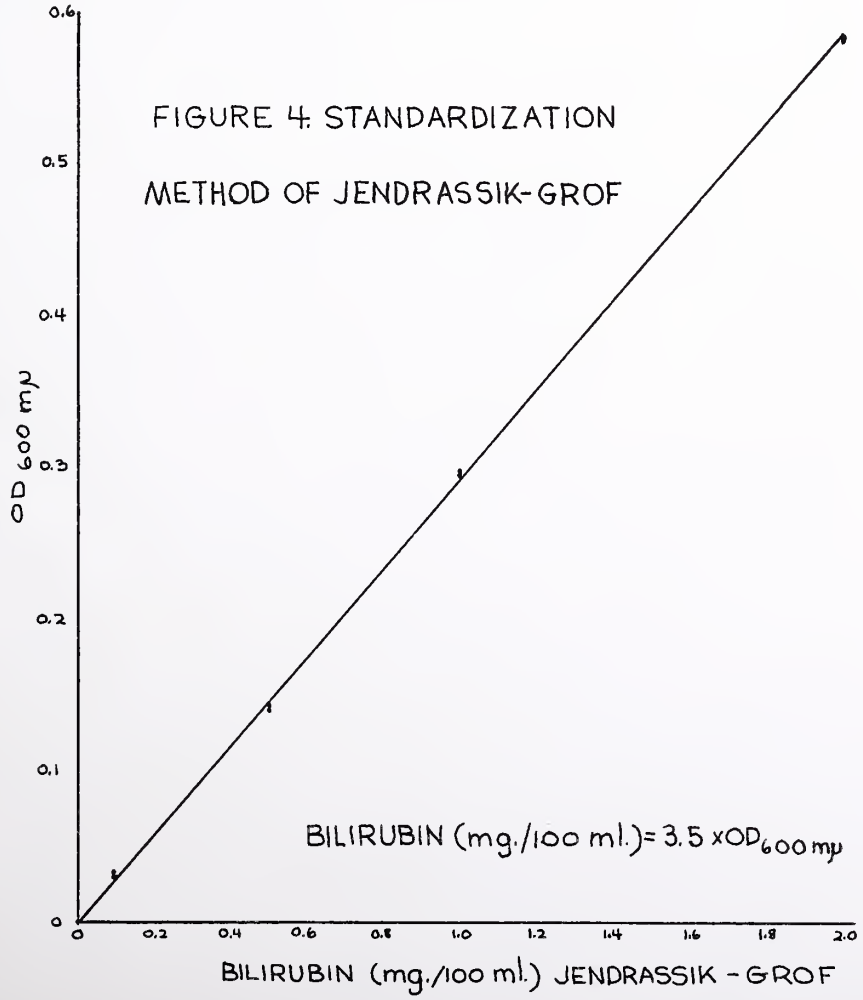


FIGURE 4. STANDARDIZATION
METHOD OF JENDRASSIK-GROF
OD 600 mμ
0.6
0.5
0.4
0.3
0.2
0.1
0
BILIRUBIN (mg./100 ml.)=3.5 xOD600 mμ
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
BILIRUBIN (mg./100 ml.) JENDRASSIK-GROF

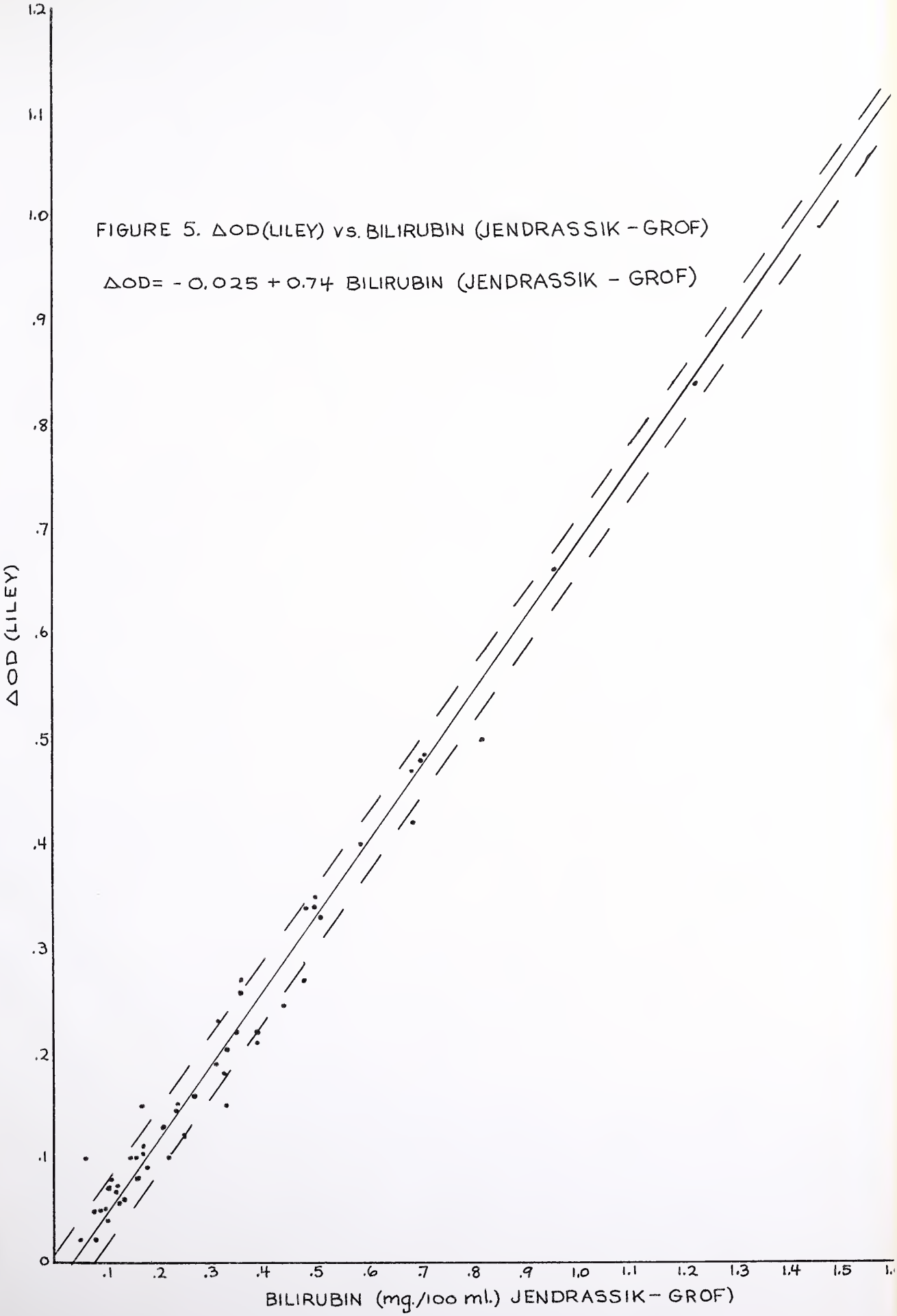


FIGURE 5. ΔOD(LILEY) vs. BILIRUBIN (JENDRASSIK - GROF)
ΔOD= - 0.025 + 0.74 BILIRUBIN (JENDRASSIK - GROF)
ΔOD (LILEY)
BILIRUBIN (mg./100 ml.) JENDRASSIK - GROF)
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
1.4
1.5

Discussion

Liley's ΔO.D. method for measuring amniotic fluid bilirubin was the first to be suggested, and it became immediately popular. Being a direct spectrophotometric method, no reagents were required. Although a continuous recording spectrophotometer is not absolutely necessary, with a manual instrument readings must be made at twelve individual wavelengths. Not only must the spectrophotometer be linear at every wavelength, it must be absolutely accurate at every wavelength. Because there is no standardization with known bilirubin samples, results must be expressed in "optical density units" and workers using two different spectrophotometers have no way of knowing whether their results are in agreement.

Walker, Fairweather and Jones (21) used a direct spectrophotometric method similar to Liley's except that per cent transmission instead of optical density was plotted against wavelength. The distance from the tangent to the curve itself at 450 mμ was measured and expressed as a difference in per cent transmission. The disadvantage of this method is that it is optical density, not per cent transmission, which is proportional to the amount of pigment present, so that when per cent transmission is used, the presence of varying amounts of turbidity in the amniotic fluid will change the estimate of bilirubin present.

Fleming and Woolf quantitated a direct spectrophotometric method for measuring bilirubin (5). They found that the four factors which contributed to the optical density of amniotic fluid were bilirubin, turbidity, and oxyhemoglobin and methemalbumin if present. By per-

forming spectrophotometric scans on these components in pure form, it was possible to find the wavelengths of greatest absorption for each of them. The optical densities of the four substances were found at each of four wavelengths, one for each component, and from the relation between these and the knowledge of the absorbance of each pure substance, the bilirubin was calculated. As with the ΔO.D. (Liley) method, the spectrophotometer must be absolutely accurate at the four wavelengths used.

Other workers have used diazo reactions for quantitating bilirubin, although some have questioned their use. Liley believed that accuracy would be decreased by dilutional errors and by deterioration of reagents (13). Freda (7) stated that chemical tests for amniotic fluid bilirubin were undesirable, since he believed that bilirubin was just one of many similar pigments from hemoglobin breakdown, all of which absorb between 400 mμ and 500 mμ, and therefore concluded that the ΔO.D. was a more accurate indication of the fetal status.

Using reverse phase Kieselguhr column chromatography, however, Brazie et al. demonstrated that the pigment in amniotic fluid is only bilirubin, so that this objection has been overcome (3).

The diazo methods that have been employed are adaptations of methods in use for serum, often with a greater proportion of amniotic fluid in the reaction mixture to intensify the final color. Although significant levels of bilirubin are often as low as 0.1 mg. per cent, protein levels are often greater than 1 gm. per cent (4), and coupling reagents that precipitate proteins may cause significant turbidity

preventing the measurement of the bilirubin present.

Robertson (19), and Watson et al. (22) used a modified Malloy-Evelyn method employing as a coupling agent methanol, which precipitates protein. Robertson also measured the direct bilirubin by omitting the coupling reagent, and by subtracting the direct from the total bilirubin, obtained the indirect which he believed was more accurate that the ΔO.D. in predicting fetal outcome. Only four poorly defined categories of fetal outcome were used, and out of a total of 252 predictions based on the ΔO.D., of which 193 were correct and 59 incorrect, 23 could be corrected by considering only the indirect bilirubin. It was not noted, however, how many correct predictions based on the ΔO.D. would be "uncorrected" by considering the indirect bilirubin, and therefore the two methods were not compared in a meaningful manner.

Michaelsson (16), using the Malloy-Evelyn method on serum, found that turbidity of the blank solution varied from time to time and from sample to sample. Other disadvantages of the Malloy-Evelyn technique are the long period, 30 minutes, required for complete diazotization and the low sensitivity. Because of the low bilirubin level in amniotic fluid compared to serum, the disadvantages of the Malloy-Evelyn method should be even more serious when applied to amniotic fluid measurements.

Robertson did not mention problems caused by turbidity of the reaction mixtures containing methanol, but this writer has found that the diazo and blank tubes often have different degrees of turbidity, making it impossible to ascribe the difference in optical density bet-

ween the two tubes as being related in any way to the amount of bilirubin present. Often the optical density due to turbidity was ten times as great as that estimated to be caused by the bilirubin. For these reasons diazotization with methanol was found to be totally unsatisfactory.

Bower and Swale employed the diazo method of Patterson, Swale and Maggs (17), in which coupling is effected by ethanol-urea solution which precipitates protein, excess diazo reagent is then inactivated by sodium azide, and finally the precipitated protein is redissolved with phenol. The resulting solutions are optically clear, but the concentrations of ethanol and phenol required are quite large, necessitating a 1:12 dilution of the amniotic fluid before the final solution to be read in the spectrophotometer is obtained; consequently, the sensitivity of the method is low. Bower and Swale expressed results for the assay of bilirubin as "positive" or "negative," and all positives were listed as having bilirubin greater than 0.12 mg. per cent, implying that levels below this value could not be accurately determined. This writer has confirmed this lack of accuracy. Repeated measurements on the same sample gave a standard deviation of 0.043, the highest of all methods tested with the exception of Robertson's. One of the difficulties was the lack of miscibility of phenol and the ethanol-water solutions, even after vigorous agitation using a mechanical mixer. When placed in the cuvette, striations would form in the solution which would not respond to all attempts at removing them, from stirring with a glass rod to blowing fine bubbles through the solution

with a Pasteur pipette. Once striations formed, optical density measurements on the solution were not repeatable.

Jendrassik and Grof developed a diazo method making use of a caffeine-sodium benzoate coupling reagent that did not precipitate protein (12). After diazotization, the reaction mixture was brought to alkaline pH, producing a blue color which was more specific for bilirubin than the pink azobilirubin formed at acid pH. Fog (6) found that the protein concentration of the sample did not affect the degree of diazotization within a fiftyfold dilution of serum. Duplicate determinations on 43 different serum samples showed a mean difference of less than 1%. The green color of the final reaction mixture was noted to be a combination of blue alkaline azobilirubin and the yellow color formed by the reaction of diazo reagent with caffeine at alkaline pH. At 600 mμ this yellow pigment absorbed a negligible amount of light, and therefore did not interfere with the bilirubin determination even in weak solutions. Michaelsson found that hemoglobin in concentrations up to 280 mg. per cent inhibited diazotization to cause an apparent loss in measured bilirubin of only 2%, which increased to 8% at a hemoglobin concentration of 1400 mg. per cent (16). The addition of ascorbic acid to the diazotization reaction mixture prevented inhibition by hemoglobin.

Gambino and Freda adapted the Jendrassik-Grof method to the measurement of bilirubin in amniotic fluid (10). The equation of the best straight line comparing ΔO.D. and bilirubin was $\Delta O.D. = -0.011 + 0.74$ Bilirubin. The coefficient of correlation for that plot was 0.99, and the molar absorptivity of azobilirubin

was 72,500, within 0.7% of that found by them. The equation of the best straight line was ΔO.D. = -0.025 + 0.74 Bilirubin. The slopes determined by this writer and Gambino are the same, 0.74, and the ΔO.D. intercepts differ by 0.014 optical density units, so that the ΔO.D. as determined by this writer is uniformly 0.01 optical density unit lower than that determined by Gambino, an insignificant difference practically.

In addition to using different coupling agents, the three diazo methods tested here employ three different types of standards. The Bower-Swale, Malloy-Evelyn, and Jendrassik-Grof methods use bilirubin standards prepared in chloroform, methanol and human serum respectively.

The Subcommittee on Bilirubin of the Standards Committee of the American Association of Clinical Chemists defined an acceptable standard (18). They decreed that standards must be prepared in fresh human serum, and that the standard must be treated in exactly the same manner as the sample whose bilirubin is to be measured.

Using the Malloy-Evelyn method, however, Meites and Traubert (15) found that standards of bilirubin in chloroform-methanol gave the same molar absorptivity as standards in aqueous serum solution, if correction was made for the volume contraction of methanol-water solutions.

The Bower-Swale standardization employs chloroform solutions of bilirubin, and also differs from the Subcommittee recommendations in that the standards and the amniotic fluid are treated differently; the reaction mixture contains acid buffer instead of amniotic fluid and bilirubin in chloroform instead of pure chloroform.

Therefore the Jendrassik-Grof method is the only one tested that as published uses standards in conformity with Subconnittee recommendations. This writer used concentrated pooled human serum albumin in the preparation of standards since fresh human serum was not available in sufficient quantities. The molar absorptivity of azobilirubin was within 0.7% of that calculated by Gambino using serum, so that pooled human albumin is shown to be a convenient acceptable source of protein.

Fleming and Woolf developed their equation by measuring the optical density of bilirubin standards in aqueous protein solutions, and the molar absorptivity of bilirubin solutions are recognized as potentially inaccurate unless they are highly purified (11).

The methods studied in this paper gave varying results in measuring bilirubin; the graphs comparing ΔO.D. with bilirubin for the Fleming-Woolf, Bower-Swale, and Jendrassik-Grof methods were 0.59, 0.66, and 0.74 respectively. This may be a reflection of the different types of standards used by each method as well as the different coupling agents used for diazotization, since varying values for bilirubin determinations in serum using different methods are will known (23).

It is essential that amniotic fluid bilirubin analyses be performed using a method which is accurate and repeatable by different workers using differing equipment. There is evidence that the ΔO.D. (Liley) method is not suitable. While it is repeatable to within 0.01 optical density unit when all measurements are made on the same spectrophotometer, varying readings may be obtained when different instruments are used. Gambino (10) found that the ΔO.D. as determined by

the Cary automatic recording spectrophotometer is consistently lower than that obtained using the Beckman DU Spectrophotometer. Robertson suggested that because the ΔO.D. method does not give consistent results when performed by different laboratories, it is probably necessary for each worker to determine his own laboratory's critical ΔO.D. values for patient management. Until these critical values have been determined, however, many fetuses would have to be poorly managed, merely in order to obtain statistics.

Many workers involved in management of erythroblastosis, furthermore, see too few patients to deduce a method of management on their own. In an attempt to deal statistically with a larger series of patients, the Ross Conference on Pediatric Research (20) gathered many workers in erythroblastosis to pool their findings. Such cooperative studies may be meaningless, however, if the ΔO.D. values are not consistent among the laboratories reporting.

Of the methods tested in this paper, the Jendrassik-Grof is most suitable for replacing direct spectrophotometry. This writer and Gambino have found its correlation with the ΔO.D. (Liley) to be 0.99, and both have obtained a value of 0.74 for the slope of the graph of ΔO.D. versus bilirubin. Of all methods tested, the Jendrassik-Grof had the lowest standard error of estimate, 0.024 optical density units. The spectrophotometer need not be of the continuous recording type, and since standards are used, it must only be linear at one wavelength.

Workers who have accumulated large quantities of data using direct spectrophotometry and who wish to use the Jendrassik-Grof method may determine their own best straight line comparing the

two methods and then convert the previously obtained ΔO.D. values into mg. per cent bilirubin, with no loss of continuity in their statistics.

With consistent and standardized techniques for measuring bilirubin, amniocentesis will be still more useful in the management of erythroblastosis.

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